Glutamate Receptor Modulation of [^{3}H]GABA Release and Intracellular Calcium in Chick Retina Cells[a]

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The retina has served in many studies as a model for studying neuronal function because it is easily accessible and because of its laminar arrangements of a limited number of basic neuron types. The retina contains almost every neurotransmitter known, but glutamate and γ-aminobutyric acid (GABA) predominate; glutamate is present in the photoreceptors and bipolar and ganglion cells,[1,2] whereas GABA is the neurotransmitter in the horizontal cells and in many amacrine cells.[1–4] Each neurotransmitter can generate various types of responses at its receptors, and, apparently, the specific glutamate and GABA transporters involved in neurotransmitter uptake are also involved in determining the synaptic activity levels. Thus, although we normally think of vesicular release of the neurotransmitters, studies with retina cells have provided evidence that the membrane transporters are important in releasing GABA.[5–9]

The existing data on the mechanism(s) of GABA release by GABAergic neurons, including retina cells, are not consistent, and at least two alternate hypotheses for GABA release have been proposed: one favoring the classical Ca^{2+}-dependent exocytotic mechanism[10,11] and another postulating the reversal of the Na^+-dependent carrier.[5,8,9,12,13] Some recent studies carried out with embryonic chick retina cells[6] called our attention to the fact that these cells apparently do not exhibit Ca^{2+}-dependent release of GABA in response to K^+ depolarization or to glutamate, suggesting that the release would be entirely nonvesicular, although contradictory reports now are found in the literature.[5,8,9,14] The presence of only Ca^{2+}-independent release of GABA in chick retina cells due to depolarization would be unequivocal evidence that nonvesicular release may constitute a physiological means for GABA release.

Therefore, we used cultured embryonic chick retina cells to study in detail the mechanism(s) of [^{3}H]GABA release stimulated by K^+ depolarization or by glutamate through its receptors, and by the specific glutamate receptor agonists, *N*-methyl-D-aspartate (NMDA), kainate (KA), quisqualate (QA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD). We studied in particular the Ca^{2+} requirement for [^{3}H]GABA release under conditions in which the carrier mediated [^{3}H]GABA release was blocked by 1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NNC-711), a specific inhibitor of the GABA carrier.[15] The NMDA receptor is permeable to Ca^{2+}, Na^+, and K^+, which will depolarize the

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membrane and thus activate voltage-sensitive Ca^{2+}-channels (VSCCs), resulting in a composite $[Ca^{2+}]_i$ signal due to Ca^{2+} influx through the receptor-associated channel and through VSCCs.[16,17] The KA and AMPA receptors are generally regarded as low Ca^{2+} permeability receptors and activation of these receptors is thought to increase the $[Ca^{2+}]_i$ mainly via the VSCCs, although some Ca^{2+} permeable KA and AMPA receptors have been described.[18–20] Also, the metabotropic glutamate receptors may increase the $[Ca^{2+}]_i$ through activation of phospholipase C, leading to the formation of inositol 1,4,5-triphosphate which releases Ca^{2+} from intracellular stores.[21]

Because the Ca^{2+} signals generated by either K^+ depolarization or by activation of glutamate receptors are diverse, they probably have distinct functions within the neurons. Thus, we considered it of importance to dissect the $[Ca^{2+}]_i$ signals generated by K^+ depolarization and by activation of each of the glutamate receptors in the same chick retina cell cultures enriched in amacrine-like neurons.[6]

EMBRYONIC CHICK RETINA CELLS DISPLAY CALCIUM-DEPENDENT RELEASE OF [^{3}H]GABA

We used monolayers of embryonic retina cells previously loaded with the fluorescent indicator Indo-1 or [^{3}H]GABA, and followed, respectively, the $[Ca^{2+}]_i$ responses and the release of [^{3}H]GABA induced by 50 mM KCl. Depolarization rapidly increased the $[Ca^{2+}]_i$ by 748.8 ± 43.8 nM (FIG. 1A), and the initial peak was followed by a slow decrease of the $[Ca^{2+}]_i$ towards a plateau at 172 ± 16.5 nM ($n = 7$) above the resting value (FIG. 1A), suggesting that the cells nearly recovered the initial $[Ca^{2+}]_i$ shortly after depolarization.[8]

The results of parallel studies show that the KCl depolarization in the absence of Ca^{2+} released about 1% of the total [^{3}H]GABA accumulated and that Ca^{2+} further increased the release of [^{3}H]GABA to over 2% release (FIG. 1B). Both the changes in $[Ca^{2+}]_i$ and the release of [^{3}H]GABA could be blocked partially by nitrendipine (0.1 μM), but ω-conotoxin GVIA (ω-CgTx; 0.5 μM), which blocked the changes in $[Ca^{2+}]_i$ by about 27%, did not influence the [^{3}H]GABA release (TABLE 1). Thus, it appears that in retina cells in culture Ca^{2+} entry through the L-type of VSCCs is coupled to the release of [^{3}H]GABA.[8]

These results lead us to conclude that depolarization of cultured retina cells with 50 mM KCl induces Ca^{2+}-dependent release of [^{3}H]GABA, in addition to carrier-mediated release (FIG. 1B). Thus, at least some of the embryonic cells of the retina have the exocytotic mechanism for releasing [^{3}H]GABA. Furthermore, the Ca^{2+}-dependent release appears to be initiated by Ca^{2+} entering through the L-type Ca^{2+} channels rather than through the N-type, which seems more common for brain tissue.

The Ca^{2+}-independent release of [^{3}H]GABA induced by K^+ depolarization can be blocked by NNC-711 (10 μM), which has been shown to block the GABA carrier.[15] In FIGURE 1B (inset), we show that, in the absence of Ca^{2+}, NNC-711 completely abolished the release of [^{3}H]GABA induced by 50 mM KCl, and that, in the presence of Ca^{2+} (1 mM), the GABA carrier blocker inhibited the release by about 66%, but did not appear to affect the Ca^{2+}-dependent release.

GLUTAMATE INCREASES THE $[Ca^{2+}]_i$ BUT STIMULATES Ca^{2+}-INDEPENDENT RELEASE OF [^{3}H]GABA

Glutamate has also been found to increase the intracellular $[Ca^{2+}]$ in several cell type cultures, but there is controversy as to whether the effect of glutamate on the

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release of [^{3}H]GABA is mediated through a rise in [Ca^{2+}].[5–9,13,14] We found that in chick retina cell cultures, which exhibit Ca^{2+}-dependent release of [^{3}H]GABA when stimulated with 50 mM KCl (see above), Ca^{2+} was inhibitory when the release was induced by glutamate (FIG. 2A). As in the case of KCl depolarization, glutamate depolarization increased the $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$; FIG. 2B) through activation of L-type VSCCs because nitrendipine inhibited the $\Delta[Ca^{2+}]_i$ by about 50%, whereas ω-CgTx was without effect.[9]

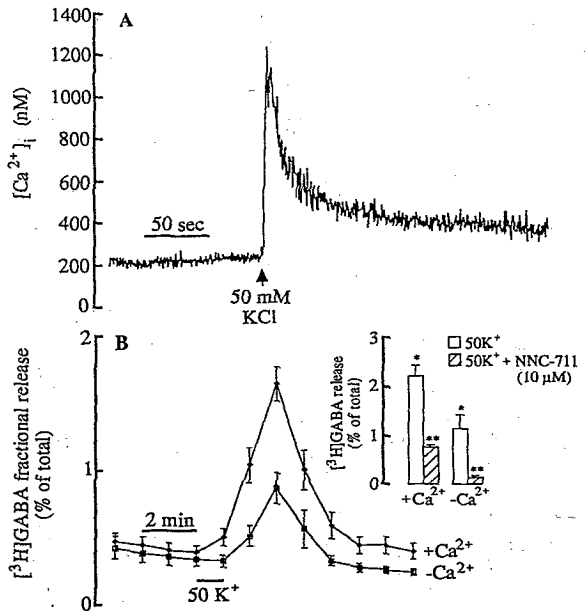

FIGURE 1. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response (A) and [^{3}H]GABA release (B) stimulated by KCl depolarization. (A) The cells were loaded with the fluorescent indicator Indo-1, and the $[Ca^{2+}]_i$ was determined as previously described.[8,9] After 4 min of preincubation in Na^+ medium (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 6 glucose, and 10 HEPES-Na, pH 7.4), the cells were stimulated with 50 mM KCl. (B) The cells were loaded with [^{3}H]GABA for 1 h at 37 °C and perfused, using a superfusion system, with Na^+ medium, with or without added Ca^{2+}. Where indicated, the cells were depolarized by replacing NaCl isoosmotically by 50 mM KCl (horizontal bar). The release of [^{3}H]GABA is expressed as previously described.[8,9] (*Inset*) Effect of NNC-711 on the release of [^{3}H]GABA induced by K^+ depolarization. When NNC-711 (10 μM) was tested, a preincubation of 1 min with the drug was performed. The data are presented as means ± SEM (n = 4–11). *Significantly different from control, $p < 0.005$.

Furthermore, the effect of glutamate on the [^{3}H]GABA release is in fact mediated through glutamate receptors because 1 μM (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,b]cyclohepten-5,10-imine maleate (MK-801) inhibited it by about 40%, and the combination of 1 μM MK-801 and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) nearly inhibited it by 100%.[9] The release of [^{3}H]GABA induced by glutamate in the presence or in the absence of Ca^{2+} was nearly all carrier mediated, as shown in FIGURE 3 depicting the effect of 10 μM NNC-711 on [^{3}H]GABA release. This drug blocked the uptake of [^{3}H]GABA in chick retina cells

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TABLE 1. Effect of Ca^{2+} Channel Blockers on $\Delta[Ca^{2+}]_i$ and [^{3}H]GABA Release Induced by KCl Depolarization[a]

	Control	Nitrendipine (0.1 μM)	ω-Cg Tx (0.5 μM)
$\Delta[Ca^{2+}]_i$ (nM)	748.8 ± 43.8 (n = 8)	265.5 ± 14.4 (n = 5)	546.5 ± 27.7 (n = 7)
[^{3}H]GABA release (% of total)	2.22 ± 0.2 (n = 11)	1.32 ± 0.11 (n = 4)	1.96 ± 0.3 (n = 7)

NOTE: The experimental conditions were similar to those described in FIGURE 1. When present, nitrendipine was applied 1 min prior to stimulation with KCl, whereas for ω-CgTx the cells were treated as described previously.[8]

[a]Adapted from Duarte *et al.*[8]

(not shown) and completely blocked the release of [^{3}H]GABA evoked by 100 μM glutamate in the absence of Ca^{2+} (FIG. 3). At 1 mM, Ca^{2+} again inhibited the release in the absence of NNC-711; in the presence of the drug the release of [^{3}H]GABA evoked by glutamate, in Ca^{2+}-containing or in Ca^{2+}-free media, was not significantly different (p = 0.097). The results suggest that relatively little or no exocytotic (Ca^{2+}-dependent) release was induced by glutamate. We studied further the Ca^{2+}-dependent release of [^{3}H]GABA mediated by the glutamate receptor agonists, NMDA, KA, QA, and AMPA, under conditions of blockade of the GABA carrier by NNC-711, to avoid carrier mediated release (see below).

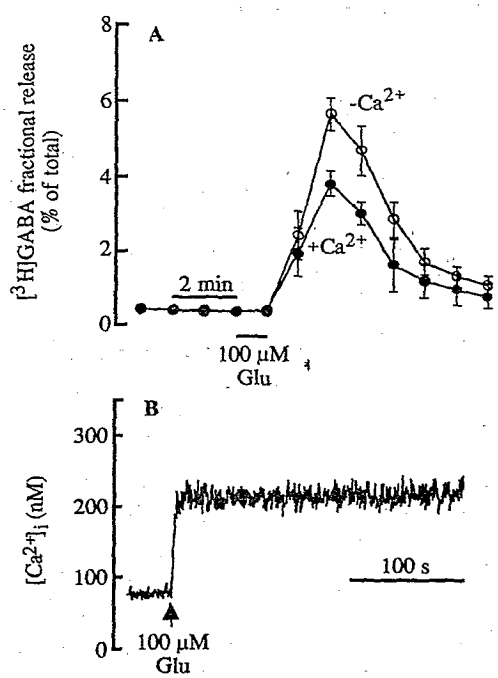

FIGURE 2. Glutamate stimulated [^{3}H]GABA release (**A**) and $[Ca^{2+}]_i$ changes (**B**) in cultured chick retina cells. (**A**) The cells were stimulated with 100 μM glutamate, as indicated by the horizontal bar, in Na^+ medium with or without added Ca^{2+}. The release of [^{3}H]GABA is expressed as previously described.[8,9] The data are presented as means ± SEM. (n = 7–8). (**B**) The cells were preincubated for 4 min in Na^+-medium and, where indicated, 100 μM glutamate was added.

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INDIVIDUAL GLUTAMATE AGONISTS INDUCE BOTH Ca^{2+}-INDEPENDENT AND Ca^{2+}-DEPENDENT RELEASE OF [^{3}H]GABA

The results described above suggest that nearly 100% of the [^{3}H]GABA release evoked by glutamate was Ca^{2+}-independent and occurs through the GABA carrier. In this section we show that stimulation of the individual ionotropic glutamate receptors, namely the NMDA and AMPA receptors, induced a significant Ca^{2+}-dependent release of [^{3}H]GABA, although most release is Ca^{2+} independent, as in the case of glutamate presented in the previous section.

Stimulation of the cells with 200 μM NMDA (under 50 mM K^+ depolarization) in the presence of glycine (3 μM) and in the absence of Ca^{2+}, produced a release of [^{3}H]GABA of about 7% per minute of the total [^{3}H]GABA accumulated, as

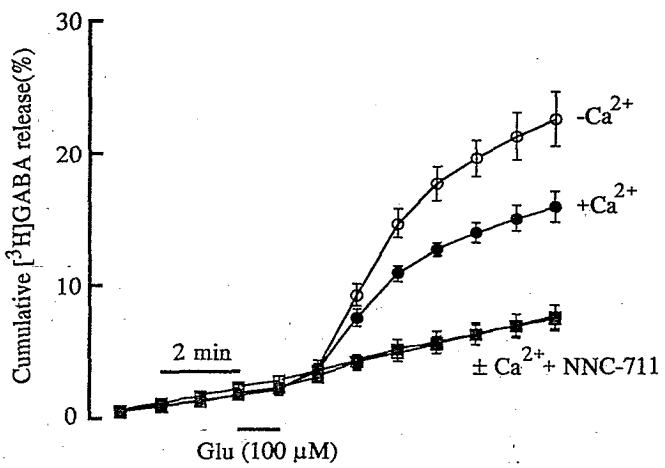

FIGURE 3. Effect of NNC-711 on the cumulative release of [^{3}H]GABA induced by glutamate (100 μM) in Na^+ medium, with or without added Ca^{2+}. When the effect of NNC-711 (10 μM) was tested, the cells were perfused with NNC-711 1 min prior to stimulation (horizontal bar) with glutamate. The release of [^{3}H]GABA is expressed as previously described.[8,9] The data are presented as means ± SEM (n = 3–8).

compared to about 1% per minute of the release produced by 50 mM KCl (FIG. 4). This effect was completely blocked by 1 μM MK-801.[9] Glutamate alone had an effect similar to that of NMDA + K^+ + glycine. Thus, either glutamate or NMDA causes massive release of [^{3}H]GABA which is Ca^{2+} independent. Similar results were obtained for KA (100 μM) and QA (10 μM), and this effect was nearly all reversed by 10 μM CNQX (FIG. 5). Thus, chick retina cells stimulated with KA released about 6.44% of the total [^{3}H]GABA/min in a Na^+ medium without Ca^{2+}, and QA released about 1.68% per minute under the same conditions. These results suggest that both agents are acting on non-NMDA receptors and that the effect is Ca^{2+} independent.

To evaluate the effect of Ca^{2+} on the release of [^{3}H]GABA evoked by the glutamate receptor agonists, we stimulated the cells with glutamate receptor agonists for short periods (1 min), in the presence and in the absence of Ca^{2+}. Short periods

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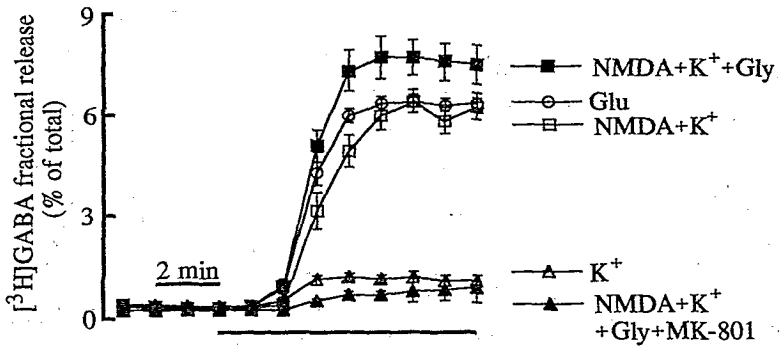

FIGURE 4. Effect of continuous stimulation with glutamate, K^+ depolarization or NMDA under K^+ depolarization on the release of [^{3}H]GABA, in the absence of added Ca^{2+}. Where indicated (horizontal bar), the superfusion solution was replaced by Na^+ medium containing 100 μM glutamate or K^+ medium prepared by isoosmotic replacement of NaCl by 50 mM KCl. When glycine (3 μM) or MK-801 (1 μM) was tested, the cells were perfused with these agents 1 min prior to stimulation with the stimulating agents. The release of [^{3}H]GABA is expressed as previously described.[8,9] The data are presented as means ± SEM (n = 3–10).

of stimulation were used in the presence of Ca^{2+} to avoid toxicity phenomena due to long exposures of the cells to glutamate.[22] Either NMDA, KA, QA, or AMPA increased the $[Ca^{2+}]_i$ in chick retina cells only if extracellular Ca^{2+} were present (not shown). The presence of Ca^{2+} significantly increased the release of [^{3}H]GABA due to stimulation by KA, QA or AMPA, but inhibited the release induced by NMDA from 7.36 ± 0.87, in the absence of Ca^{2+}, to 4.05 ± 0.39% in the presence of Ca^{2+} (TABLE 2). All agonists increased the $[Ca^{2+}]_i$ (see below), but apparently only in the case of NMDA (and glutamate) does the increase in $[Ca^{2+}]_i$ produce inhibition of [^{3}H]GABA release (TABLE 2).

The effect of Ca^{2+} on the release of [^{3}H]GABA can best be studied if we inhibit the contribution of the GABA carrier to the release. We did this by inhibiting the

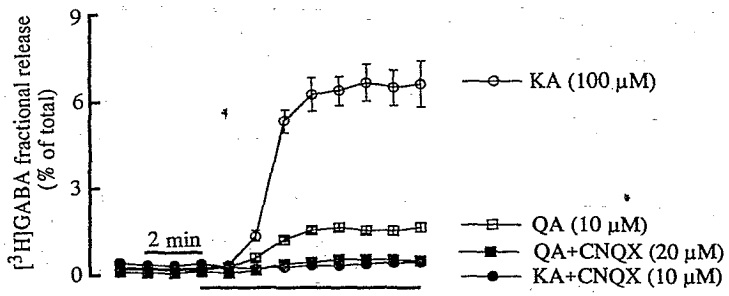

FIGURE 5. Effect of CNQX on the release of [^{3}H]GABA, evoked by kainate (KA) (100 μM) or quisqualate (10 μM). Experiments were performed as described in FIGURE 4, in the absence of added Ca^{2+}. When CNQX (10 or 20 μM) was tested, the cells were perfused with the antagonist 1 min prior to stimulation with KA or QA. The release of [^{3}H]GABA is expressed as previously described.[8,9] Results are presented as means ± SEM (n = 3–5).

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carrier with NNC-711,[15] which totally inhibits glutamate-induced release in the absence of external Ca^{2+} (FIG. 3). In the presence of Ca^{2+}, we found that NNC-711 (10 μM) did not block all [^{3}H]GABA release induced by the glutamate agonists, and that apparently NMDA, QA, and AMPA, but not KA, caused Ca^{2+}-dependent release of [^{3}H]GABA in the presence of NNC-711 (FIG. 6).

It is of interest to note that the overall effect of Ca^{2+} on the release evoked by NMDA is a reduction in the release of [^{3}H]GABA (TABLE 2), but the exocytotic component (Ca^{2+}-dependent) is actually stimulated by Ca^{2+} (FIG. 6). This may mean that the effect of Ca^{2+} on the release is complex and is exerted not only on the exocytotic component. Evidence for this is also obtained from the influence of Ca^{2+} on the KA effect; thus, FIGURE 6 and TABLE 2 show clearly that Ca^{2+} increases the total release of [^{3}H]GABA induced by KA from a value of 13.23 ± 0.80% in the absence of Ca^{2+} to a value of 18.03 ± 0.63% in the presence of Ca^{2+}. Nevertheless, when we blocked the carrier with NNC-711, KA increased [^{3}H]GABA release to about the same extent in the presence or in the absence of Ca^{2+} (FIG. 6). Thus, it would appear that KA, under conditions in which the carrier is blocked, causes

TABLE 2. Effect of Glutamate Receptor Agonists on the $[Ca^{2+}]_i$ and [^{3}H]GABA Release[a]

Agonist	$\Delta[Ca^{2+}]_i$ (nM)	[^{3}H]GABA Release (%)	
		Ca^{2+} Absent	Ca^{2+} Present
NMDA	156.60 ± 8.25 (n = 12)	7.36 ± 0.87 (n = 7)	4.05 ± 0.39 (n = 4)
KA	276.51 ± 6.78 (n = 9)	13.23 ± 0.80 (n = 8)	18.03 ± 0.63 (n = 7)
QA	93.94 ± 5.76 (n = 8)	3.57 ± 0.33 (n = 13)	4.88 ± 0.52 (n = 10)
AMPA	120.36 ± 6.00 (n = 7)	4.41 ± 0.75 (n = 4)	7.07 ± 0.88 (n = 5)

[a]The experiments were performed in Na^+ medium as described in the legends to FIGURES 6 and 8. The release of [^{3}H]GABA was obtained in the absence or presence of added Ca^{2+}, without NNC-711, and for 1 min of stimulation with the various agonists.

release through a mechanism which involves neither the carrier nor the exocytotic (Ca^{2+}-dependent) mechanism.

Calcium increases the release of [^{3}H]GABA stimulated by either QA or AMPA, in the presence (FIG. 6) or in the absence of NNC-711 (TABLE 2), suggesting that a Ca^{2+}-dependent release occurs in the absence of carrier-mediated release. The effect on the exocytotic release is more clearly seen in the case of AMPA (50 μM), a more specific agonist for the ionotropic QA receptor. AMPA increased the $[Ca^{2+}]_i$ by 120 nM (TABLE 2) and evoked a release of 7.07 ± 0.88% of the total [^{3}H]GABA in the presence of Ca^{2+}, whereas only 4.41 ± 0.75% was released in the absence of Ca^{2+} (TABLE 2), and a substantial release remained in the presence of NNC-711, which was dependent on Ca^{2+} (FIG. 6).

Although QA stimulates both the ionotropic and the metabotropic receptors, (1S,3R)-ACPD, the agonist of metabotropic ACPD receptor, did not influence the release of [^{3}H]GABA, in the presence or in the absence of Ca^{2+}, suggesting that this receptor was not involved (results not shown).

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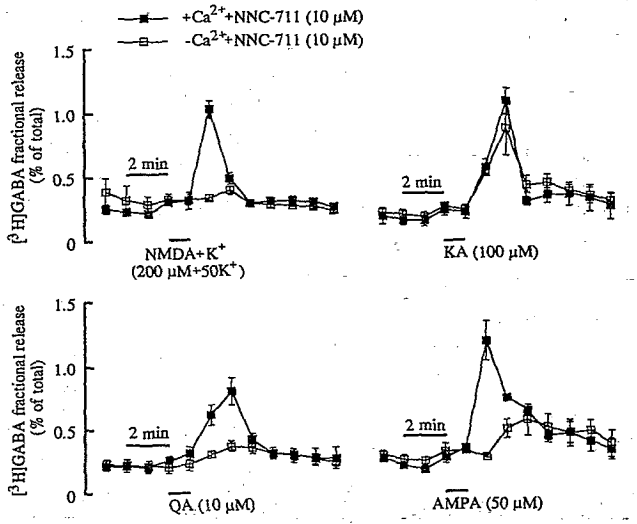

FIGURE 6. Effect of Ca^{2+} on [^{3}H]GABA release evoked by glutamate receptor agonists (NMDA, KA, QA, and AMPA), in the presence of NNC-711. The cells were stimulated with NMDA (200 μM) under K^+ depolarization, KA (100 μM), QA (10 μM) or AMPA (50 μM), and a preincubation with NNC-711 (10 μM) was made prior to stimulation with the agonists. The release of [^{3}H]GABA is expressed as previously described.[8,9] Results represent means ± SEM (n = 3–5).

MECHANISMS OF $[Ca^{2+}]_i$ REGULATION BY GLUTAMATE RECEPTOR AGONISTS

The NMDA receptor is permeable to Ca^{2+} and is expected to generate composite $[Ca^{2+}]_i$ signals due to the influx of Ca^{2+} through the receptor-associated channel and through VSCCs.[16] The KA and AMPA receptors are regarded as low-Ca^{2+} permeability receptors,[16,17] and activation of these receptors is thought to increase the $[Ca^{2+}]_i$ mainly via depolarization-induced opening of the VSCCs, although some Ca^{2+}-permeable KA and AMPA receptors have been described.[18–20] The $[Ca^{2+}]_i$ may also rise due to mobilization of intracellular Ca^{2+} stores because of activation of phospholipase C through the metabotropic glutamate receptors.[21]

The different effects of Ca^{2+} on the release of [^{3}H]GABA reported in the previous section may be related to the fact that the Ca^{2+} signals provided by activation of the glutamate receptors are diverse. Thus, it is important to separate the $[Ca^{2+}]_i$ signals generated by each glutamate receptor into its component parts. Our results show in fact that the agonists of the ionotropic glutamate receptors, NMDA, KA and AMPA, increased the $[Ca^{2+}]_i$ because of composite responses comprising Ca^{2+} entering through the receptor channels and through VSCCs.

Calcium Entry through Glutamate Receptors

To evaluate the contribution of the receptor-associated channels to the $[Ca^{2+}]_i$ response to glutamate, NMDA, KA, and AMPA, the cells were stimulated with each

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agonist in a Na^+-free *N*-methyl-D-glucamine (NMG) medium. When glutamate was added under these conditions, the effect on the rise in $[Ca^{2+}]_i$ was decreased to about 38% the value obtained in a Na^+ medium (FIG. 7A and B). Also nitrendipine was able to decrease the $[Ca^{2+}]_i$ response induced by glutamate in a Na^+ medium by about 53% (FIG. 7A), which suggests that both VSCCs and glutamate receptor-associated channels allow Ca^{2+} entry upon glutamate receptor stimulation.

In Mg^{2+}-free Na^+ medium, 100 μM NMDA evoked a sustained increase in $[Ca^{2+}]_i$ of 156.6 ± 8.2 nM that could be reversed by MK-801 (FIGS. 8A and 9A). Even in the presence of 1.4 mM Mg^{2+}, NMDA increased the $[Ca^{2+}]_i$ to a peak of about 77 nM which then declined to a plateau of about 32 nM above the resting concentration. Furthermore, when the extracellular Na^+ was replaced by NMG, the response to NMDA in the absence of Mg^{2+} was about 30 nM (FIG. 8B), which corresponds to the contribution of the NMDA receptor-associated channels to the $[Ca^{2+}]_i$ response.

Addition of 100 μM KA to the medium increased the $[Ca^{2+}]_i$ by 276 nM and then decreased to a plateau at about 74% of the value of the peak (FIG. 8C); the effect could be partially blocked by 20 μM CNQX to a value of 66 nM (FIG. 9B). When the extracellular Na^+ was replaced by NMG, the effect of KA on the $[Ca^{2+}]_i$ rise decreased to about 60% of the value obtained in the Na^+ medium, probably because of the absence of entry of Ca^{2+} through the VSCCs in the NMG medium (FIG. 8C). In the case of AMPA the receptor-associated channel seems to have a much smaller contribution to the $[Ca^{2+}]_i$ rise than that observed for the KA receptor (FIG. 8D). Activation of the AMPA receptors increased the $[Ca^{2+}]_i$ by 120 nM, but this effect was only slightly affected by CNQX (FIG. 9C).

The effect of (1S,3R)-ACPD on the $[Ca^{2+}]_i$ was evaluated in the retina cells, and we found that 200 μM (1S,3R)-ACPD did not affect the $[Ca^{2+}]_i$ in the presence (not shown) or in the absence of Ca^{2+} (FIG. 10). The presence of (1S,3R)-ACPD receptors in chick retina cells was further evaluated by measuring the accumulation of myo-[^{3}H]inositol phosphates evoked by two minutes of stimulation with 200 μM (1S,3R)-ACPD. The agonist increased the accumulation of [^{3}H]InsP_1, [^{3}H]InsP_2, and

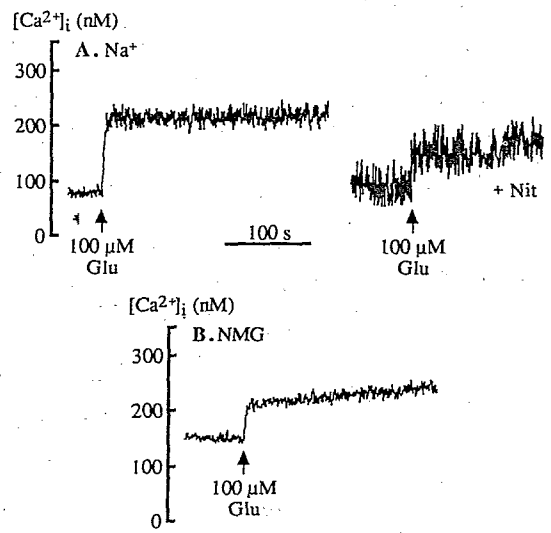

FIGURE 7. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) responses to glutamate in Na^+- or *N*-methyl-D-glucamine (NMG) media. The cells were preincubated for 4 min in Na^+ (A) or NMG (B; containing 132 mM NMG, but otherwise identical to the Na^+ medium) media, and, where indicated, 100 μM glutamate was added. In A the control response to glutamate (*left*) is compared to the effect after 4 min of preincubation with 1 μM nitrendipine.

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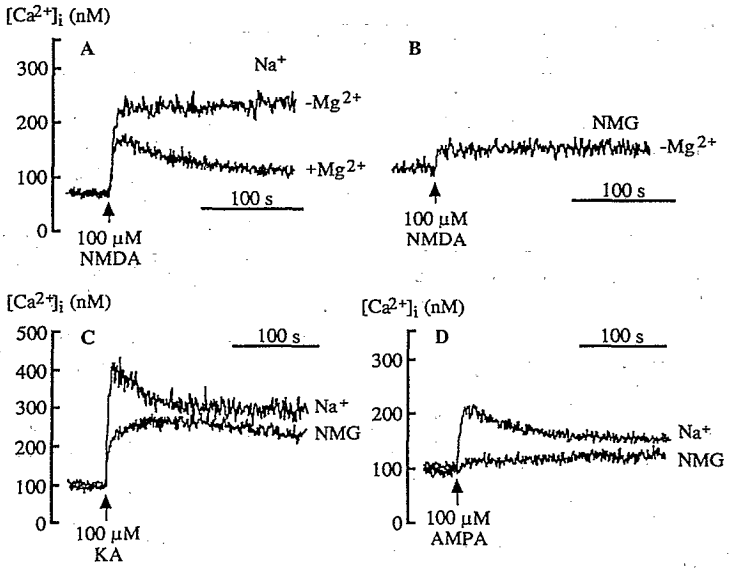

FIGURE 8. Effect of glutamate receptor agonists on the $[Ca^{2+}]_i$ in Na^+ and NMG media. The cells were stimulated with 100 µM NMDA (**A** and **B**), 100 µM KA (**C**) or 100 µM AMPA (**D**), after 4 min of preincubation in the indicated media. The response to NMDA was tested in Na^+ medium (**A**), with ($+Mg^{2+}$) or without ($-Mg^{2+}$) 1.4 mM $MgCl_2$, or in Mg^{2+}-free NMG medium (**B**).

[^{3}H]InsP_3, but this effect was not antagonized by L-AP3 (100–500 µM), a selective antagonist of some metabotropic receptors[21] (not shown).

Ca^{2+} Entry through Voltage-Sensitive Ca^{2+} Channels

The results reported in FIGURE 9 show that a large fraction of the Ca^{2+} entering the retina cells, as a result of glutamate agonists stimulation, passes through channels sensitive to nitrendipine. Indeed, nitrendipine (1 µM) decreased by 76 nM, 51 nM, and 55 nM the $[Ca^{2+}]_i$ increase evoked by NMDA, KA, and AMPA, respectively (FIG. 9A–C), suggesting that L-type channels are present in these cells. The P-type Ca^{2+} channels, identified by their sensitivity to ω-Aga IVA, also contribute significantly to the response to KA, whereas ω-Aga IVA was without effect on the $[Ca^{2+}]_i$ transients evoked by NMDA and AMPA (FIG. 9A–C). It is of interest to note that the glutamate agonists do not activate N-type VSCCs[9] although we showed previously that these channels are present in the retina cell cultures used in the experiments.[8]

The differential activation of VSCCs by NMDA, KA, and AMPA may reflect (1) that the receptors are localized in different cells which may or not contain P-type Ca^{2+} channels and that the L-type channels are always present or (2) that the receptors and the P- and N-type Ca^{2+} channels may have a heterogenous distribution within the cells.

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Potentiation by TPA of the Ca^{2+} Entry through the KA Receptor-associated Channel

We took advantage of the high Ca^{2+} permeability of the KA receptor in our preparation to study the effect of protein kinase C (PKC) on the activity of the receptor. The PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 200 nM)

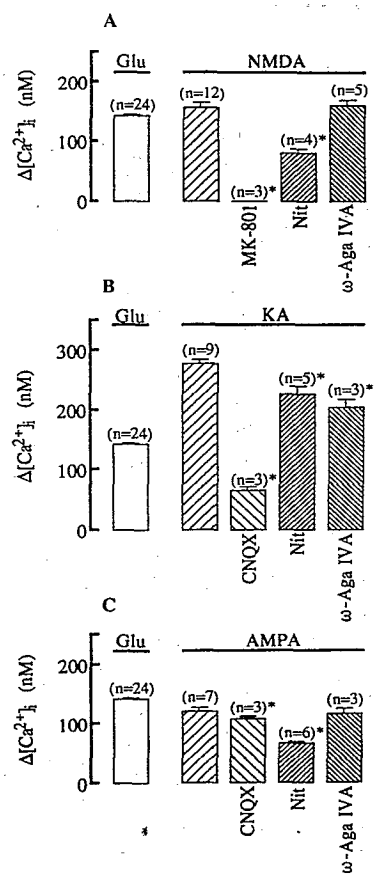

FIGURE 9. Effect of voltage-sensitive Ca^{2+} channel (VSCC) antagonists and glutamate receptor antagonists on the $[Ca^{2+}]_i$ responses to NMDA, KA or AMPA. The average initial $[Ca^{2+}]_i$ responses ($\Delta[Ca^{2+}]_i$) to 100 μM NMDA (**A**) in Mg^{2+}-free Na^+ medium, 100 μM KA (**B**) or 100 μM AMPA (**C**) in Na^+ medium, are compared with the effect of 100 μM glutamate (Glu), determined as indicated in FIGURE 2B. The effects of nitrendipine (Nit; 1.5 μM), MK-801 (7.5 μM), CNQX (20 μM), and ω-Aga IVA (200 nM) were determined after 4 min of preincubation with the antagonists. When ω-Aga IVA was tested, the cells were preincubated with the toxin for 1 h, during the loading with Indo-1. The effect of NMDA was always tested in Mg^{2+}-free Na^+ medium, whereas the $[Ca^{2+}]_i$ response to glutamate, kainate, and AMPA were determined in the presence of Mg^{2+} (Na^+ medium). Results are expressed as means ± SEM of the indicated number of experiments, carried out in different preparations. *Significantly different from control, $p < 0.05$.

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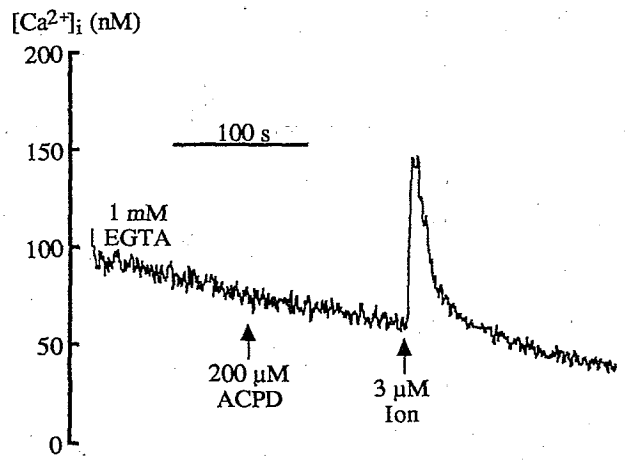

FIGURE 10. Lack of effect of (1S,3R)-ACPD on the $[Ca^{2+}]_i$. The cells were preincubated for 2 min in Ca^{2+}-free (1 mM EGTA) Na^+ medium, and, where indicated, 200 µM ACPD and 3 µM ionomycin were added. The trace is representative of experiments performed in three independent preparations.

increased the $[Ca^{2+}]_i$ response to KA in NMG medium to about 130% of the control (FIG. 11). Conversely, TPA decreased the effect of KA in Na^+ medium to about 80% of the control (FIG. 11). To test whether this inhibition was due to a decreased Ca^{2+} entry through VSCCs, we studied the effect of TPA on the $[Ca^{2+}]_i$ response to KCl depolarization, which is expected to specifically activate VSCCs. After a 4-min pre-incubation with TPA, the effect of 40 mM KCl depolarization was inhibited by about 25% (FIG. 12). Thus, although the activity of the Ca^{2+} permeable KA receptors is increased by TPA, the overall $[Ca^{2+}]_i$ response in Na^+ medium is inhibited because of the effect of the phorbol esters on the VSCCs.

DISCUSSION AND CONCLUSIONS

[^{3}H]GABA Release by Potassium Depolarization

In the present work, we clearly show that K^+ depolarization activates L-type Ca^{2+} channels coupled to Ca^{2+}-dependent [^{3}H]GABA release in embryonic chick retina cells. Inasmuch as [^{3}H]GABA seems to be accumulated in horizontal and amacrine cells,[1,3,6] these cells must be the source of the [^{3}H]GABA release. However, toad (*B. marinus*) and goldfish horizontal cells have only Ca^{2+}-independent [^{3}H]GABA release,[5,23] and these cells do not have synaptic vesicles.[3] Therefore, the Ca^{2+}-dependent release by our preparation may represent release by amacrine cells, although Hofmann and Möckel[6] were unable to show Ca^{2+}-dependent release in a similar chick retina culture. These authors did not use a superfusion system to determine release, and it is possible that other substances released, which would accumulate in the medium, could be interfering with the Ca^{2+}-dependent (exocytotic) release.

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Although most studies have shown only Ca^{2+}-independent release of [^{3}H]GABA by various preparations of retina,[6,10,23,24] at least one report shows that goldfish retina have a "small but significant" Ca^{2+}-dependent release of [^{3}H]GABA.[5] Our studies also show that, in accordance with results from other retina cell preparations, a large fraction of the [^{3}H]GABA release induced by K^+ depolarization is Ca^{2+}-independent (FIGS. 1 and 3) and represents the release of cytoplasmic [^{3}H]GABA through the GABA carrier because it can be blocked by NNC-711 (FIGS. 1 and 3).

Release of [^{3}H]GABA by Glutamate and Glutamate Agonists

One of the first observations in our studies was that Ca^{2+} decreases the release of [^{3}H]GABA induced by glutamate (FIGS. 2 and 3). It is of interest to note that about 50% of the Ca^{2+} entry induced by glutamate uses the same L-type channels which become active because of K^+ depolarization and, in this case, increases [^{3}H]GABA release (TABLE I). There is no clear explanation for this observation especially

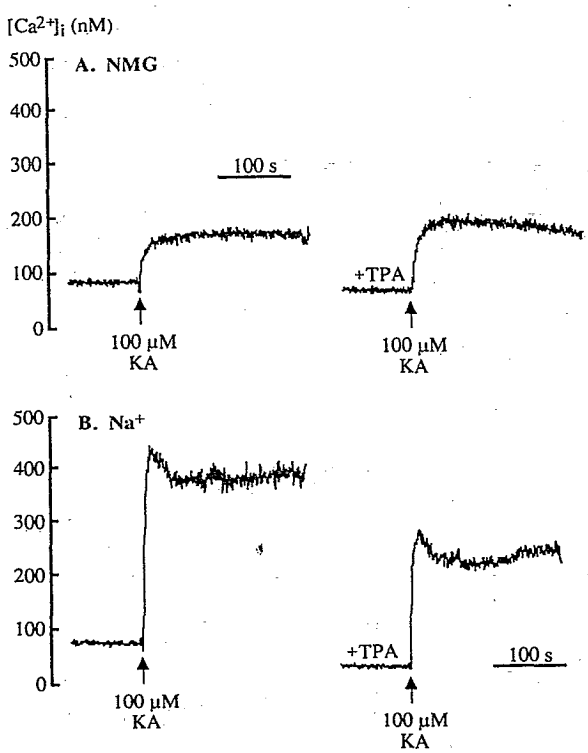

FIGURE 11. Effect of TPA on the $[Ca^{2+}]_i$ response to kainate, in NMG (**A**) or Na^+ (**B**) media. The cells were preincubated for 4 min in the indicated media, with (+TPA) or without 200 nM TPA, and, where indicated, 100 µM KA was added. The traces are representative of experiments carried out in duplicate in 6–8 different preparations.

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because in both cases activation of L-type VSCCs accounts for about 50% of the Ca^{2+} entry.

The coupling of the glutamate receptors to the Ca^{2+} channels requires Na^+ in the external medium, and the $[Na^+]_i$ actually increases due to glutamate stimulation (results not shown). Our results are in accordance with earlier reports by Sucher *et al.*[25] that Ca^{2+} antagonists attenuate the rise in $[Ca^{2+}]_i$ due to glutamate in rat retinal ganglion cells, and other reports exist showing that activation of the glutamate receptors (NMDA, KA, QA) elevates the $[Ca^{2+}]_i$ indirectly by depolarizing the membrane, which then activates the Ca^{2+} influx through VSCCs.[16,18,19]

The [^{3}H]GABA release stimulated by glutamate or NMDA was more pronounced in the absence of Ca^{2+} and could be nearly totally blocked by NNC-711 (FIGS. 3 and 6A and TABLE 2). Similar Ca^{2+}-independent release of [^{3}H]GABA has been reported in other cell systems,[6,13,23,26] and in all cases Na^+ is required, which

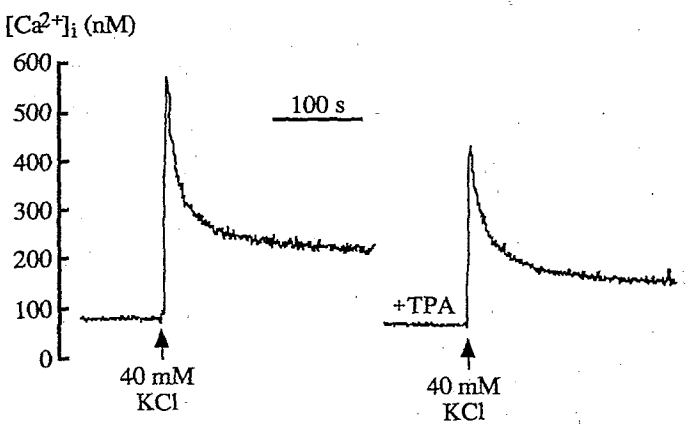

FIGURE 12. Inhibition by TPA of the K^+-stimulated $[Ca^{2+}]_i$ increase. The experiments were performed as indicated in FIGURE 1A, and where indicated (+TPA) the cells were preincubated for 4 min with 200 nM TPA. The traces are representative of duplicate experiments carried out in eight different preparations.

suggests that an increase in $[Na^+]_i$ coupled to membrane depolarization reduces the electrochemical Na^+ gradient causing reversal of the GABA carrier.

In conclusion, glutamate increases the $[Ca^{2+}]_i$ of chick retina cells and induces Na^+-dependent and Ca^{2+}-independent release of [^{3}H]GABA, which is carrier mediated. The presence of Ca^{2+} actually inhibits the total release of [^{3}H]GABA induced by glutamate, although the mechanism for this effect of Ca^{2+} is not clear. In the absence of carrier-mediated release of [^{3}H]GABA, Ca^{2+} does not affect significantly the release of [^{3}H]GABA induced by glutamate. It should be noted that most glutamate receptors may be located far from the nerve terminals, as has been suggested for chick motoneurons,[27] and in these cases the [^{3}H]GABA release may be mediated by the GABA carrier located on the soma or dendrites of the GABAergic retina cells. This localization of GABA has already been reported.[7]

The results of the studies in which we stimulated each of the glutamate receptors

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with specific agonists (NMDA, KA, QA, and AMPA) are in accordance with previous reports that retinal cells in culture express NMDA and non-NMDA receptors, both of which can mediate Ca^{2+}-independent release of [^{3}H]GABA.[6] We further showed that these cells also exhibit Ca^{2+}-dependent release when stimulated by the glutamate receptor agonists NMDA, QA, and AMPA (FIG. 6), when the studies are carried out in the presence of NNC-711, to inhibit the carrier-mediated release.

When we studied the total release of [^{3}H]GABA, which includes carrier-mediated release and Ca^{2+}-dependent release, Ca^{2+} had an overall inhibitory effect (FIG. 3 and TABLE 2) when either glutamate or NMDA were the stimulating agents; however, this effect was not observed for the other agonists (TABLE 2). Furthermore, in the case of KA, QA or AMPA, Ca^{2+} increased the total release (TABLE 2), and QA and AMPA, as well as NMDA, induced Ca^{2+}-dependent release of [^{3}H]GABA when the carrier was blocked (FIG. 6). The Ca^{2+} inhibition of the [^{3}H]GABA release induced by glutamate or NMDA may be related to the effect of Ca^{2+} on the binding of glutamate or NMDA to the receptors.

Mechanisms of $[Ca^{2+}]_i$ Regulation by Glutamate Receptor Agonists

Glutamate probably is the main excitatory neurotransmitter activating amacrine cells,[1,2] and the results reported here show that the agonists of the ionotropic glutamate receptors, NMDA, KA, QA, and AMPA, increase the $[Ca^{2+}]_i$ in cultures enriched in amacrine cells. The increase in $[Ca^{2+}]_i$ was found to be due to Ca^{2+} entry through the receptor-associated channels and the VSCCs, as has been reported for other cell types.[16]

The $[Ca^{2+}]_i$ increases due to Ca^{2+} entry through the NMDA receptor-associated channel, as determined in Mg^{2+}-free NMG medium, was about 25% of the total response to the agonist in Na^+ medium, which included the VSCCs contribution. Permeation of Ca^{2+} through the NMDA receptor-associated channel has been reported for several cell types,[28] but the non-NMDA ionotropic glutamate receptors generally exhibit low Ca^{2+} permeability.[18–20]

Our results show clearly that, in retina cells, the influx of Ca^{2+} through the KA receptor-associated channel was higher than that observed for the NMDA receptor-associated channel (FIG. 9); the influx of Ca^{2+} through the KA receptor-associated channel corresponds to about 60% of the initial $[Ca^{2+}]_i$ response in a Na^+ medium, and it is potentiated by activation of PKC with TPA (FIG. 11A). However, the overall effect of PKC activation in a normal Na^+ medium is to inhibit the increase in $[Ca^{2+}]_i$ induced by KA (FIG. 11B), because PKC activation has an inhibitory effect on the VSCCs triggered by KA depolarization (FIG. 12). Activation of PKC has previously been shown to modulate the activity of glutamate receptors[29,30] and of VSCCs.[31] The influx of Ca^{2+} through the KA receptor-associated channel is a Ca^{2+} pathway that may be of great interest in mediating excitatory amino acid induced phenomena of various types, particularly when NMDA receptors are not present.[32,33] Moreover, the influx of Ca^{2+} through the KA receptor is assumed to contribute to the KA-induced neurotoxicity in some neurons *in vitro*.[18] We found that domoic acid activates the KA receptor and triggers exocytotic release of [^{3}H]GABA.[34]

Cultured retina cells have at least three pharmacologically distinct classes of VSCCs: L-type, sensitive to dihydropyridines;[8,35] N-type, sensitive to ω-CgTx;[8,9] and, as shown in this work, also the presence of P-type VSCCs sensitive to ω-Aga IVA,[36] when the cells are stimulated with KA (FIG. 9), but not detected when the stimulation is with NMDA or AMPA. The P-type channels have also been reported

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for horizontal cells of the bass retina.[37] It should be noted that although the N-type VSCCs are present in our preparation, as detected under K^+ depolarization,[8] they do not contribute to the $[Ca^{2+}]_i$ responses to glutamate.[9]

The differential activation of the VSCCs by NMDA, KA, and AMPA (FIG. 9) may reflect that (1) the receptors may be localized in different cells, all of which would contain the L-type VSCCs, but only some cell types would contain the P-type of channels or (2) the receptors and the P- and N-type channels are distributed heterogeneously in the cells.

Finally, (1S,3R)-ACPD increased intracellular accumulation of myo-[^{3}H]inositol phosphates (not shown), but had no effect on $[Ca^{2+}]_i$ (FIG. 10). Similar results have been reported for other cell preparations.[29,38,39] The increase in the accumulation of inositol phosphates without affecting the $[Ca^{2+}]_i$ suggests that in these cells the important signal may be diacylglycerol. The activation of PKC by diacylglycerol may modulate the activity of other glutamate receptors and the influx of Ca^{2+} through VSCCs.

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